New Phytologist

Research

Combined transcriptome and translatome analyses reveal a role for tryptophan-dependent auxin biosynthesis in the control of *DOG1*-dependent seed dormancy

Bing Bai[1,2], Ondřej Novák[3], Karin Ljung[3], Johannes Hanson[1,4]* and Leónie Bentsink[1,2]*

[1]Molecular Plant Physiology, Institute of Environmental Biology, Utrecht University, 3584 CH Utrecht, the Netherlands; [2]Wageningen Seed Laboratory, Laboratory of Plant Physiology, Wageningen University, 6708 PB Wageningen, the Netherlands; [3]Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden; [4]Umeå Plant Science Center, Department of Plant Physiology, Umeå University, SE-901 87 Umeå, Sweden

Authors for correspondence:
Leónie Bentsink
Tel: +31 317 48 13 25
Email: leonie.bentsink@wur.nl

Johannes Hanson
Tel: +46 90 786 67 44
Email: johannes.hanson@umu.se

Received: *7 December 2016*
Accepted: *7 October 2017*

New Phytologist (2018) **217:** 1077–1085
doi: 10.1111/nph.14885

Key words: *Arabidopsis thaliana*, auxin, polysome profiling, ribosome, seed dormancy, seed germination, transcription.

Summary

- The importance of translational regulation during Arabidopsis seed germination has been shown previously. Here the role of transcriptional and translational regulation during seed imbibition of the very dormant *DELAY OF GERMINATION 1* (*DOG1*) near-isogenic line was investigated.
- Polysome profiling was performed on dormant and after-ripened seeds imbibed for 6 and 24 h in water and in the transcription inhibitor cordycepin. Transcriptome and translatome changes were investigated.
- Ribosomal profiles of after-ripened seeds imbibed in cordycepin mimic those of dormant seeds. The polysome occupancy of mRNA species is not affected by germination inhibition, either as a result of seed dormancy or as a result of cordycepin treatment, indicating the importance of the regulation of transcript abundance.
- The expression of auxin metabolism genes is discriminative during the imbibition of after-ripened and dormant seeds, which is confirmed by altered concentrations of indole-3-acetic acid conjugates and precursors.

Introduction

Seed dormancy, defined as the inability of a viable seed to germinate under optimal conditions, is an important adaptive trait for plants to survive in nature (Bewley, 1997). Seed dormancy is a complex trait for which substantial natural genetic variation is present in *Arabidopsis thaliana*. *DELAY OF GERMINATION 1* (*DOG1*) is the major quantitative trait locus underlying this natural genetic variation (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006, 2010; Huang *et al.*, 2010). The gene encodes a protein with unknown but conserved function across plant species (Graeber *et al.*, 2014). The strong dormancy phenotype of the Cape Verde Islands (Cvi) allele of *DOG1* provides an ideal model system to investigate molecular pathways regulating seed dormancy.

Abscisic acid and GAs are two important hormones antagonistically regulating seed dormancy and germination (Koornneef *et al.*, 2002; Liu *et al.*, 2010). Seed dormancy can be relieved by after-ripening, which refers to a period of seed dry storage after seed harvest. During this period, transcription and metabolism are limited, because of the low moisture content and small nuclear size caused by chromatin condensation (Fait *et al.*, 2006; van Zanten *et al.*, 2011; Gao *et al.*, 2013; Meimoun *et al.*, 2014). Dry dormant and after-ripened seeds hardly show any difference in transcript patterns; however, transcriptional changes become visible when the seeds imbibe (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007). Moreover, differences in the abundance of individual proteins have been reported between dormant and after-ripened dry seeds (Chibani *et al.*, 2006). Post-transcriptional regulation is proposed to regulate seed dormancy release, possibly through mRNA oxidation (Bazin *et al.*, 2011; El-Maarouf-Bouteau *et al.*, 2013), which inhibits protein translation *in vitro* (Bazin *et al.*, 2011). Here, we investigated the role of transcription and translation during the imbibition of dormant and after-ripened seeds, making use of polysomal profiling and transcription inhibitors. Polysomal profiling uses a sucrose gradient-based fractionation method for separation of mRNAs based on their association with polysomes and thus identifies mRNAs that are being translated. These mRNAs can be identified by high-throughput profiling techniques such as microarray analysis and RNA sequencing (Mustroph *et al.*, 2009b; Layat *et al.*, 2014; Lin *et al.*, 2014; Vragovic *et al.*, 2015). The ratio between polysomal bound mRNA and total mRNA of a specific mRNA

*These authors contributed equally to this work.

represents the polysome occupancy (PO) of that mRNA (Bai *et al.*, 2016). Translation is primarily regulated at the initiation level, and therefore polysomal binding is a relevant proxy for translational activity or protein synthesis (Browning and Bailey-Serres, 2015). The importance of translational control during seed germination has previously been shown (Basbouss-Serhal *et al.*, 2015; Bai *et al.*, 2016). Here we tested the importance of translational control during *DOG1*-dependent dormancy and showed different transcriptional patterns in dormant and after-ripened seeds during seed imbibition. Moreover, inhibition of germination by *DOG1* and the transcriptional inhibitor cordycepin affected transcript abundance of genes involved in tryptophan (Trp)-dependent auxin and indole glucosinolate pathways.

Materials and Methods

Plant material and seed germination conditions

The *Arabidopsis thaliana* near-isogenic line carrying the Cvi introgression at the position of *DOG1* in a Landsberg *erecta* genetic background (NIL*DOG17-1*; here referred to as NIL*DOG1*) was originally introduced by Alonso-Blanco *et al.* (2003) and was used in the current study for its high dormancy behavior. NIL*DOG1* plants were grown in three biological replicates, with four plants for each replicate, and seeds were harvested at maturity. Half of the freshly harvested dormant seeds were stored directly at −80°C to retain dormancy and the other half were after-ripened in ambient conditions (20–25°C and 40–60% relative humidity) for dormancy release. Seed germination was scored following the after-ripening until 100% germination was reached. Germination experiments were performed as described previously (Joosen *et al.*, 2010). Six samples of *c.* 50–150 seeds were spread on wetted papers using a mask to ensure accurate spacing. Piled-up trays were wrapped in a closed transparent plastic bag. The experiment was carried out in a 22°C incubator under continuous light (143 μmol m^{-2} s^{-1}). Pictures were taken once a day for a period of 6 d using a Nikon D80 camera (Nikon, Tokyo, Japan) fixed to a repro stand with a 60 mm macro objective. The camera was connected to a computer using Nikon Camera Control Pro software, v.2.0. Clustering of seeds was prevented as much as possible. Germination was scored using the Germinator package (Joosen *et al.*, 2010). The effect of transcription inhibition on seed germination was tested by germinating the completely after-ripened seeds on different dosages (0.1, 1, 10, 100 and 1000 μM) of the transcription inhibitors cordycepin and α-amanitin (Sigma). Seed germination and seedling establishment were evaluated every day. For the ribosome isolation, both dormant and after-ripened seeds were spread on wetted papers filled with either 1 mM cordycepin or water to ensure homogeneous spacing. The Petri dishes were wrapped with parafilm M (Bemis Company Inc., Neeneah, WI, USA) to prevent water loss during seed imbibition. The experiment was carried out in a 22°C incubator under continuous light (143 μmol m^{-2} s^{-1}). Seeds were sampled at 6 and 24 h after the start of imbibition (HAI). The harvested tissue was frozen in liquid nitrogen followed by freeze-drying. The dry material was stored at −80°C for further analyses.

Isolation of total RNA and polysomal RNA and polysome analysis

For the isolation of polysomal RNA, 400 mg of freeze-dried tissue was extracted with 8 ml of polysome extraction buffer (PEB; 400 mM Tris, pH 9.0, 200 mM KCl, 35 mM $MgCl_2$, 5 mM EGTA, 50 μg ml^{-1} cycloheximide, 50 μg ml^{-1} chloramphenicol) according to Subramanian (1978) and Mustroph *et al.* (2009a) with some modifications. The extracts were loaded on top of a sucrose cushion (1.75 M sucrose in PEB) and centrifuged (18 h, 90 000 ***g***) using a Beckman Ti70 rotor for 18 h (Beckman Coulter, Brea, CA, USA). The resulting pellet was resuspended in wash buffer (200 mM Tris, pH 9.0, 200 mM KCl, 0.025 M EGTA, 35 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg ml^{-1} cycloheximide, 50 μg ml^{-1} chloramphenicol), loaded on a 20–60% linear sucrose gradient, and centrifuged at 190 000 ***g*** for 1.5 h at 4°C using the Beckman SW55 rotor (Beckman Coulter). After ultracentrifugation, the gradients were fractionated into 20 fractions using a Teledyne Isco Density Gradient Fractionation System (Teledyne Isco. Lincoln, NE, USA) with online spectrophotometric detection of 254 nm. The polysomal fractions were pooled for polysomal RNA isolation. The ribosome abundance is reflected by the area under the curve and was calculated after subtracting the baseline obtained by measuring a blank gradient and normalizing to total area under the curve to account for possible uneven loading of the gradients.

Microarray hybridization and data analysis

Affymetrix Arabidopsis Gene 1.1 ST Arrays (Affymetrix, Santa Clara, CA, USA) were hybridized using the GeneChip® 3′ IVT Express kit (Affymetrix; cat. no. 901229) according to the manufacturer's instructions. Hybridization data were analyzed and gene-specific signal intensities were computed using the R statistical programming environment (www.R-project.com), the BioConductor package affy (Gautier *et al.*, 2004) and the Brainarray cdf file v.17.1.0 (http://brainarray.mbni.med.umich.edu/). DNA microarray data are available in Supporting Information Table S1 and in the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE75368. The limma and affy packages were used for RMA normalization (Irizarry *et al.*, 2003) Probe set intensity signals that never exceeded the noise threshold (logExprs < 4 in all samples) were removed. Signal distribution before and after RMA normalization and RNA degradation were evaluated using the affy package (Fig. S1). A linear model and Empirical Bayes methods were applied to assess differential expression (Smyth, 2005; Diboun *et al.*, 2006). Gene enrichment analyses (Tables S2–S4) were performed using PANTHER Overrepresentation Test (release 20160715, *P*-value = 0.05) from the Gene Ontology Consortium (http://geneontology.org/page/go-enrichment-analysis).

Auxin metabolite profiling

Seeds were imbibed for 6 or 24 h, harvested in liquid nitrogen and freeze-dried; 1 mg freeze-dried material per replicate was

used for IAA metabolite profiling as described previously (Novak *et al.*, 2012), with minor modifications (Novak *et al.*, 2016). Two-way ANOVA and Tukey honest significant differences test were performed in R (v.13.4.0) for each metabolite at a significance level of 0.05.

Results and discussion

Polysomal profiling reveals translational inhibition in dormant seeds

Seeds of NIL*DOG1*-Cvi (Cape Verde Islands) were used to investigate the importance of translation during the imbibition of dormant seeds. The germination capacity of NIL*DOG1* seeds was followed during after-ripening. Freshly harvested seeds do not germinate, but dry storage (after-ripening) releases dormancy and the germination frequency gradually increases until it reaches 100% after 120 d (Fig. 1a). The freshly harvested dormant (D) and fully after-ripened nondormant seeds (AR) that present fully contrasting dormancy levels were used to determine the role of translation in dormant imbibed seed, using a translatomics approach. For that, polysomal profiles were run on dormant and after-ripened seeds at 6 and 24 HAI. At these time points, seeds do not yet show visible germination (Fig. 1b). The ribosomal profiles reveal no differences between dormant and after-ripened seeds at 6 HAI; however, at 24 HAI after-ripened seeds show a larger proportion of polysomes than do dormant seeds, indicating that after-ripened seeds are actively translating (Figs 1c,d, S2).

Inhibition of germination in dormant seeds is controlled by regulation of transcript abundance

The increase of polysomes in after-ripened seeds 24 HAI could be a result of increased specific translation of mRNAs or increased levels of translatable mRNAs. Both aspects play roles during germination and seedling establishment in Arabidopsis (Bai *et al.*, 2016). To investigate this during seed dormancy regulation, total and polysomal-associated mRNAs of dormant and after-ripened seeds were compared using microarray analysis. This methodology does not allow a distinction to be made between changed transcription and changed mRNA stability. Therefore, genes that were expressed to relatively higher levels in dormant or after-ripened seeds during imbibition were defined as dormancy- or germination-associated genes, respectively. At 6 HAI, 304 and 300 genes were dormancy-associated and 315 and 258 genes were germination-associated in the total and polysomal mRNA fractions, respectively. At 24 HAI, numbers increased to 991 and 1136 for the dormancy-associated genes and 1691 and

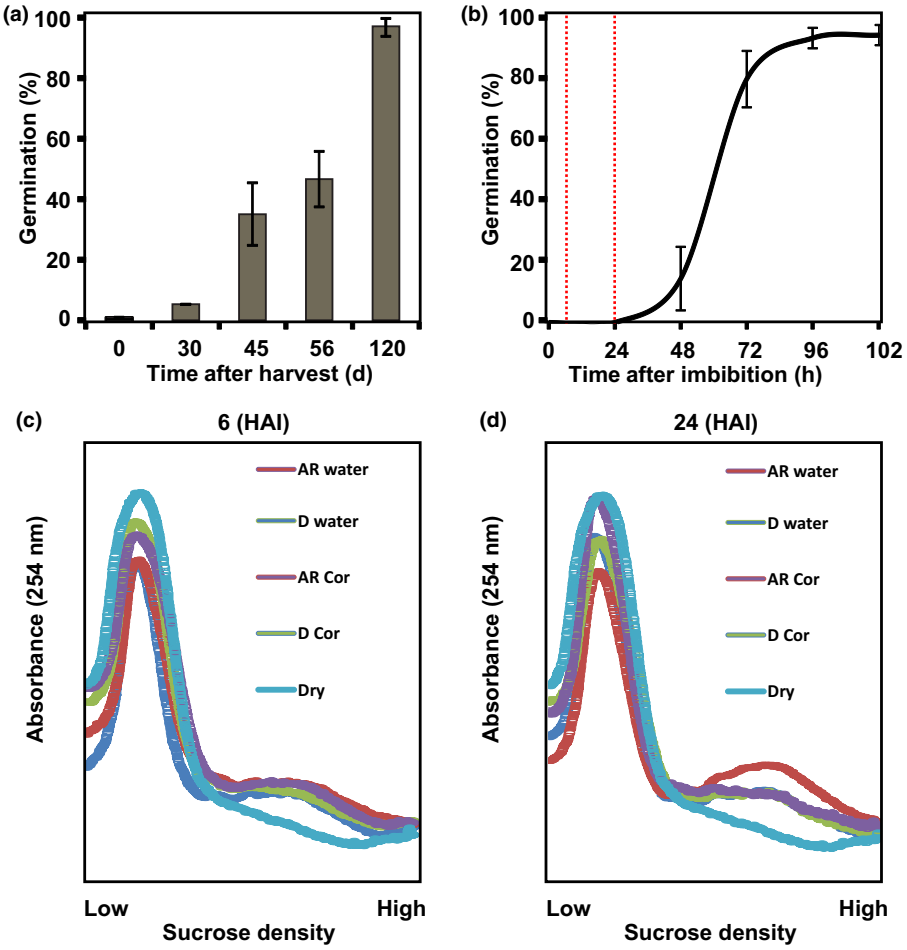


Fig. 1 Germination behavior of Arabidopsis NIL*DOG1* seeds following after-ripening. (a) Seed maximum germination determined after harvest at different times following after-ripening. (b) Germination dynamics of seeds after dormancy release. Red dashed lines represent the two imbibition stages, at 6 and 24 h after the start of imbibition (HAI), at which the transcriptome and translatome are compared. The data in (a) and (b) are presented as means ± SD of three independent replicates. (c) Polysome profiles of dormant (D) and after-ripened (AR) seeds at 6 HAI in both water and cordycepin (Cor). (d) Polysome profiles of D and AR seeds after at 24 HAI in both water and cordycepin (Cor). The data in (c) and (d) are presented as means of three independent replicates.

1789 genes for the germination-associated genes in total and polysomal fractions, respectively (Fig. 2a). To address whether translational regulation occurs during the imbibition of dormant seeds, the PO for each mRNA was analyzed (relative level of mRNA in the polysomal fraction compared with its level in the total RNA pool). Our analysis showed that the dormancy state hardly affected the PO of the individual mRNAs (Fig. 2a). This is confirmed by the highly significant correlation between the change of specific mRNA species in the total mRNA in dormant vs after-ripened seeds and the change of the same mRNA in the polysomal mRNA (Fig. 2b). Overall, these analyses show that the observed increased translation in after-ripened seeds (Fig. 1d) depends on transcription and that the impact of translational regulation is relatively minor. This finding contrasts with conclusions of Basbouss-Serhal *et al.* (2015), where translational regulation during the imbibition of after-ripened and dormant Columbia seeds was suggested. The discrepancy observed may be caused by the different experimental systems used. We define dormancy release based on after-ripening time and use fully discriminative stages (zero (D) vs 100% germination (AR)), while previously Basbouss-Serhal *et al.* (2015) studied temperature-dependent germination that leads to a germination difference of 40% between the two dormancy stages. In addition, we imbibed the seeds in light, whereas the seeds in the study of Basbouss-Serhal *et al.* (2015) were imbibed in darkness. Light signaling has previous been shown to greatly impact translation in seedlings (Juntawong & Bailey-Serres, 2012; Gamm *et al.*, 2014; Missra *et al.*, 2015). Thus, the discrepancy in the results may be related to the different light or temperature conditions (Leon & Owen, 2003; Hofmann, 2014) or to other differences in experimental conditions, and further illustrates the complex multifactorial regulation of seed germination (Bentsink & Koornneef, 2008; Rajjou *et al.*, 2012).

Role of transcription during the imbibition of dormant and after-ripened seed

Transcriptional regulation in the control of seed dormancy is important and was further investigated using transcriptional inhibitors. For this, two transcriptional inhibitors were used, α-amanitin and cordycepin. α-Amanitin was reported to inhibit seedling establishment but not germination, whereas cordycepin fully inhibited germination (Rajjou *et al.*, 2004). In our study, both transcriptional inhibitors had a dosage-dependent effect on seed germination and seedling growth (Fig. 3a,b); however, 1 mM cordycepin completely abolished seed germination and seedling establishment. The difference in inhibitory effect between α-amanitin and cordycepin might be a result of differential uptake of these compounds by seeds. Cordycepin (1 mM) was used to inhibit transcription because it effectively blocked seed germination. Interestingly, the polysomal profiles of the cordycepin-treated seeds mimic those of the dormant seeds (Fig. 1c,d). Next, the effect of cordycepin on the total and polysomal-associated mRNAs was investigated. The number of genes differentially expressed at 6 HAI in cordycepin vs water is relatively low; 12 genes and one gene are induced and 19 and seven genes are inhibited by cordycepin on the total and polysomal mRNA levels, respectively (Figs 3, S3). This indicates that cordycepin hardly affects transcription at 6 HAI. Cordycepin blocks RNA polymerase II during mRNA chain elongation and it can also interfere with RNA 3′-end formation and polyadenylation, as suggested by Holbein *et al.* (2009). The lack of effect in early seed germination may be because cordycepin has not (yet) penetrated the seed or because the post-transcriptional effects of cordycepin dominate in the early stages as a result of limited transcriptional activity at this time point. At 24 HAI these differences have increased to 168 and 218 induced genes and 332 and 300

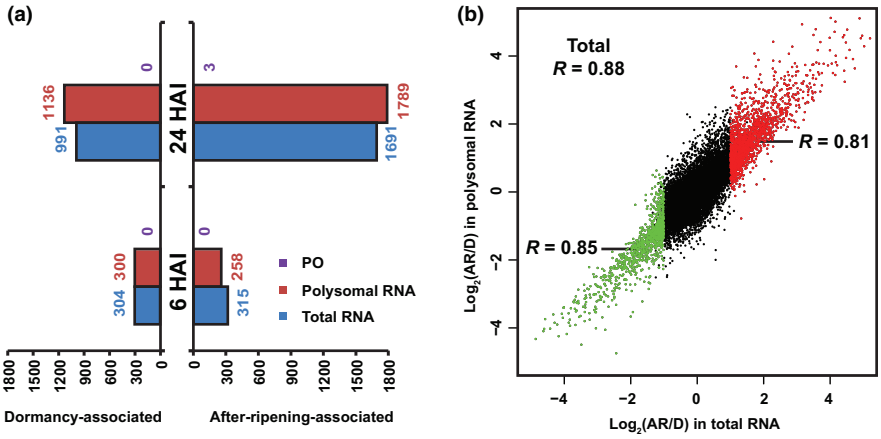


Fig. 2 The influence of dormancy level on the abundances of total mRNA and polysomal mRNA in Arabidopsis seeds. (a) The effect of dormancy on total RNA abundance (blue bars), polysomal RNA abundance (red bars) and polysome occupancy (PO = polysomal RNA abundance/total mRNA abundance; purple bars) at two stages of imbibition (6 and 24 h after the start of imbibition, HAI). Genes are considered as transcriptionally or translationally regulated when $\log_2$(fold change) > 1 and $P < 0.05$ adjusted by the false discovery rate. Dormancy-associated genes are genes that are higher in dormant (D) than in after-ripened (AR) seeds, and after-ripening-associated genes are those that are higher in AR than in D seeds. (b) Correlation between total RNA changes ($\log_2$AR/D) and polysomal RNA changes ($\log_2$AR/D) of genes transcriptionally after-ripening-associated (red dots) and transcriptionally dormancy-associated (green dots) in AR seeds compared with D seeds at 24 HAI. Black dots represent genes that are not associated with either of the earlier-mentioned categories. Correlation coefficients are highly significant ($P < 2.2E{-}16$).

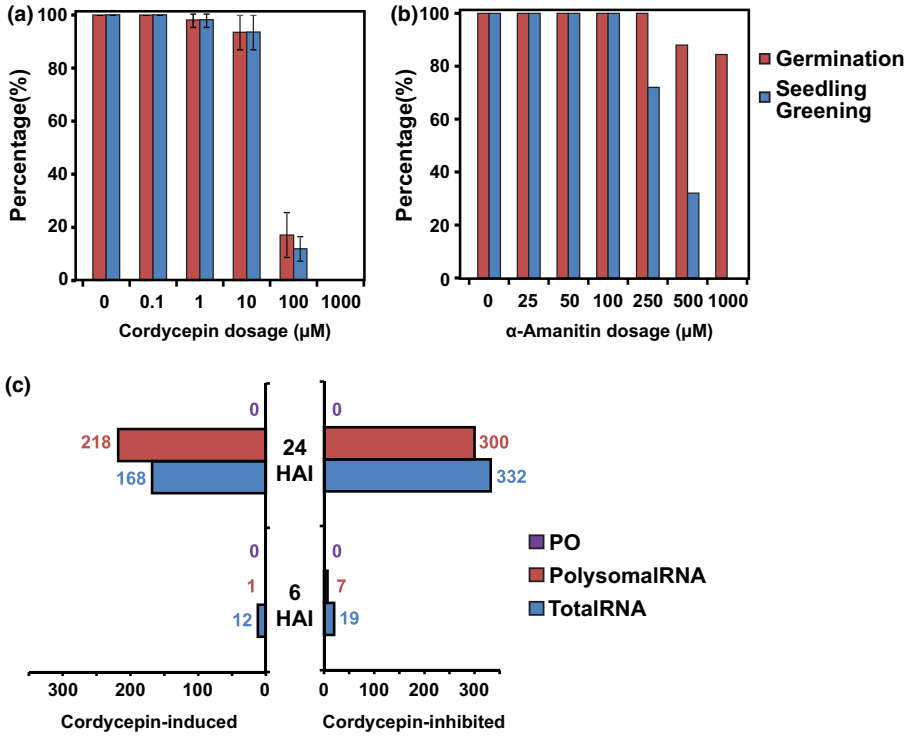
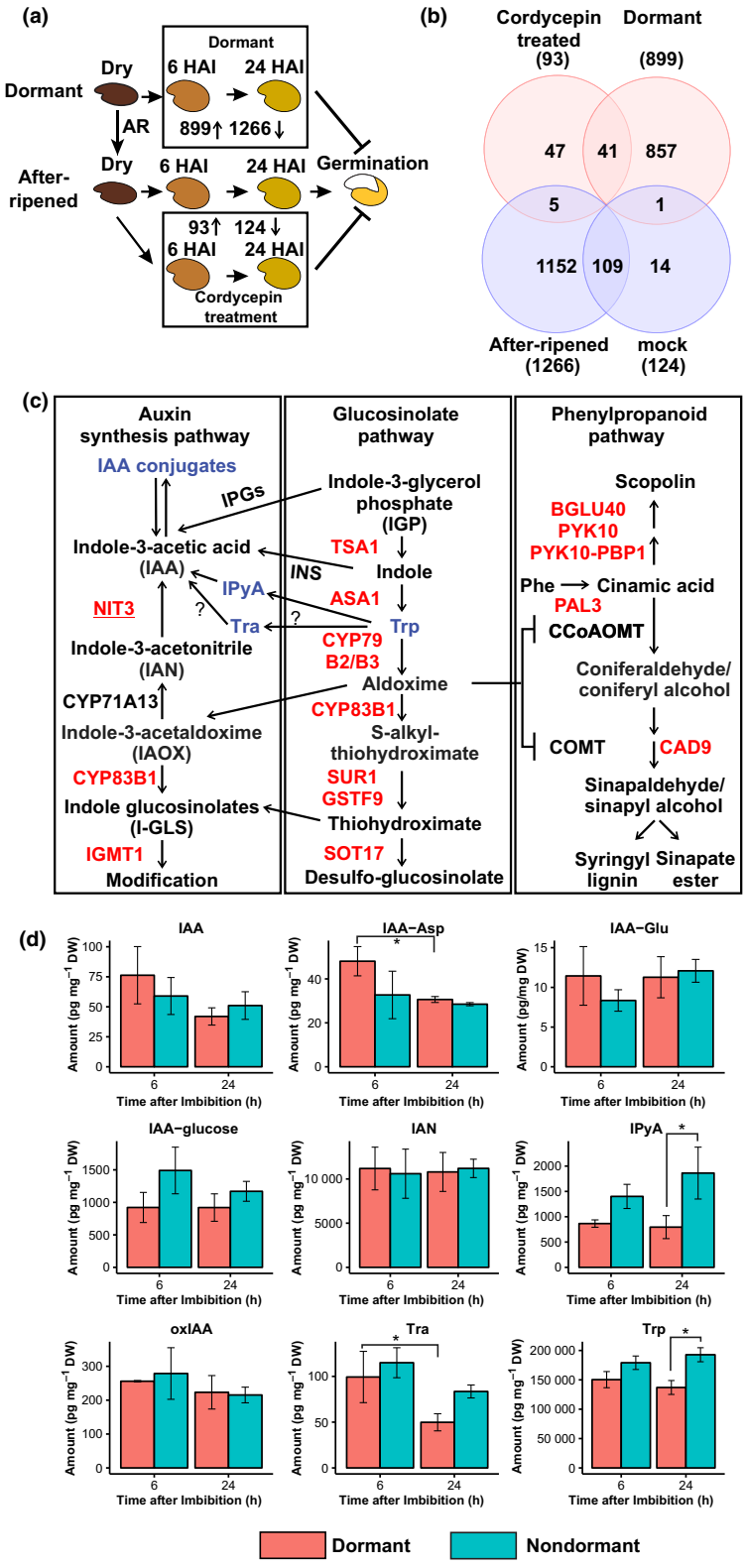


Fig. 3 Cordycepin effect on transcription during two stages of Arabidopsis seed imbibition. (a) Dosage-dependent germination inhibition by cordycepin. Bars represent means ± SD of three biological replicates. (b) Dosage-dependent germination inhibition by α-amanitin. (c) Number of transcripts influenced and inhibited by cordycepin in after-ripened seeds at 6 and 24 h after the start of imbibition (HAI) in total RNA and polysomal RNA, respectively. PO, polysome occupancy.

inhibited genes in the total and polysomal associated mRNAs, respectively. We also provided the data for the cordycepin-treated dormant seeds; however, as these seeds cannot be separated phenotypically from the water-treated seeds (both are nongermination) we do not discuss these genes further. Although transcription is expected to be blocked by cordycepin, up-regulation of genes is observed. This suggests that cordycepin does not completely block transcription. Another explanation could be that the total pool of mRNA is reduced as a result of mRNA decay and that the genes identified as up-regulated are genes that are more stable. Investigating this possibility is of special interest, as a role for mRNA decay in the regulation of seed dormancy has recently been reported (Basbouss-Serhal *et al.*, 2017). As an effect of global mRNA decay is hard to reveal using microarrays, as a result of global normalization, we performed quantitative reverse transcription polymerase chain reaction experiments for a selected group of genes (Table S7) that are revealed as up-regulated after cordycepin treatment (Fig. S5). The analyses confirmed the microarray data. The reference genes that we used for the normalization showed a stable expression and we therefore exclude an effect of global mRNA decay in these analyses. Overall, the minor effect of the inhibition of transcription at 6 HAI suggests that transcriptional activity at early time points does not determine whether seeds are able to germinate or not.

Role of the Trp-dependent auxin biosynthesis pathway in germination inhibition

In the presence of the transcriptional inhibitor cordycepin, dormant and after-ripened seeds do not germinate. As no PO changes were detected when comparing after-ripened (germinating) and nongerminating (dormant and cordycepin-inhibited) seeds, the overlap between the dormant and cordycepin-inhibited transcriptomes was investigated. Time-dependent changes from 6 to 24 HAI in dormant, nongerminating seeds were compared with the same time-dependent changes in after-ripened, germinating seeds. These analyses resulted in four groups of genes: those that are either up- or down-regulated from 6 to 24 HAI in the dormant seeds and those that are up- or down-regulated from 6 to 24 HAI in the after-ripened seeds. These four groups were compared with each other to select the genes that are dormancy-associated (899 genes that are dormancy up-regulated and after-ripening down-regulated from 6 to 24 HAI) and those that are after-ripening-associated (1266 genes that are after-ripening up-regulated and dormancy down-regulated from 6 to 24 HAI) (Fig. 4a). Gene ontology analysis revealed that the dormancy-associated genes are related to abiotic stress responses such as light, heat, oxidative stimulus and seed development, while a series of metabolic processes were after-ripening-associated (Table S3). Similarly, time-dependent changes from 6 to 24 HAI in cordycepin-treated seeds were compared with the same time-dependent changes in after-ripened, germinating seeds. These analyses resulted in four groups of genes: those that are either up- or down-regulated from 6 to 24 in the cordycepin-treated seeds and those that are up- or down-regulated from 6 to 24 in the after-ripened seeds. These four groups were compared with each other to select the genes that are cordycepin-associated (93 genes that are cordycepin up-regulated and mock (imbibition of after-ripened seeds in water) down-regulated from 6 to 24 HAI) and those that are mock-associated (124 genes that are mock up- and cordycepin down-regulated from 6 to 24 HAI) (Fig. 4a). Among



the cordycepin-associated genes were the hormone metabolism genes *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 5, 6* (*NCED5*, *6*) and *GIBBERELLIN 20-OXIDASE 3 (GA20OX3)*, confirming the importance of ABA and GA in the control of germination. Sulfur and indole-derived biosynthetic processes were mock-associated (Table S3). The overlap between the differentially expressed genes of the two types of germination inhibition experiments (after-ripened vs dormant seeds and after-ripened vs cordycepin-treated seeds) was significant (Fig. 4b; 41 genes up and 109 genes down in nongerminating seeds). This large overlap

Fig. 4 The effect of after-ripening and cordycepin on the transcriptional changes from 6 to 24 h after the start of seed imbibition (HAI). (a) Dormancy and cordycepin are two independent factors that block seed germination in Arabidopsis (indicated by the blunt-ended lines). The upwards and downwards arrows indicate the dormancy- and after-ripening-associated genes or cordycepin- and mock (after-ripened seeds imbibed in water)-associated genes during 6–24 HAI. (b) Venn diagram comparing the genes influenced by dormancy and cordycepin during 6–24 HAI. (c) The enriched metabolic pathway for the genes influenced by both cordycepin and dormancy during seed imbibition. The genes presented in red are dormancy- and cordycepin-associated genes, and the underlined gene (NIT3) was associated with cordycepin only. The genes in black encode enzymes catalyzing metabolic conversions that were not detected in the current study. The metabolites in blue are significantly changed either by dormancy or during seed imbibition. The blunt-ended lines indicate inhibition of the pathway. The question mark indicates a likely, but unconfirmed, conversion. GSTF9, glutathione S-transferase PHI 9; SOT17, sulfotransferase 17; SUR1, SUPERROOT 1; IGMT1, indole glucosinolate o-methyltransferase 1; TSA1, tryptophan synthase alpha chain; ASA1, anthranilate synthase alpha subunit 1; BGLU40, beta-glucosidase 40; PYK10, beta-glucosidase 23; CAD9, cinnamyl alcohol dehydrogenase 9; PAL3, phenyl alanine ammonia-lyase 3; PBP1, PYK10-binding protein 1; NIT3, NITRILASE3; IPGs, indole-3-glycerol-phosphate synthases; INS, indole synthase; CCoAOMT, caffeoyl-CoA O-methyltransferase; COMT, caffeic acid O-methyltransferase; Trp, tryptophan; Phe, phenylalanine. (d) Level of free IAA, IAA conjugates and IAA synthesis pathway intermediates during dormant and after-ripened seed imbibition. Ant, anthranilate; Trp, L-tryptophan; Tra, tryptamine; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, *trans*-/*cis*-indole-3-acetaldoxime; IPyA, indole-3-pyruvic acid; IAA-glc, IAA-glucose; IAAsp, IAA-aspartate; IAGlu, IAA-glutamate; oxIAA, 2-oxoindole-3-acetic acid; oxIAA-glc; oxIAA-glucose. Data are normalized by seed DW. Error bars ± SE (*, $P < 0.05$, tested by two-way ANOVA and Tukey honest significant differences test).

further strengthens our conclusion that seed dormancy is mainly controlled at the transcript level.

Interestingly, many of the overlapping genes are related to both Trp-dependent auxin (IAA) and glucosinolate biosynthesis (Fig. 4c; Table S4). Trp-dependent auxin biosynthesis is linked to the indole glucosinolate pathway through the intermediate indole-3-acetaldoxime (IAOx; Zhao *et al.*, 2002; Sugawara *et al.*, 2009; Ljung, 2013). Several genes related to these pathways were down-regulated in the transcriptome of nongerminating seeds (Fig. 4c; Table S5). This includes transcripts coding for enzymes sequentially involved in the conversion of indole-3-glycerol phosphate (IGP) to indole (Ouyang *et al.*, 2000), indole to Trp (Sun *et al.*, 2009), Trp to IAOx (Zhao *et al.*, 2002), and IAOx to S-alkyl-thiohydroximate and thiohydroximate (Barlier *et al.*, 2000; Bak & Feyereisen, 2001; Klein & Papenbrock, 2009; Pfalz *et al.*, 2011; Kim *et al.*, 2015). Further, the glucosinolate biosynthesis pathway probably interacts with the phenylpropanoid biosynthetic pathway through IAOx (Hemm *et al.*, 2003; Mach, 2015). Also enzymes involved in different steps in the phenylpropanoid pathway are down-regulated in nongerminating seeds (Fig. 4c; Table S5). These findings suggest that Trp-dependent auxin biosynthesis and related pathways are strongly repressed in nongerminating (dormant and transcriptionally inhibited) seeds.

To investigate whether the repression of Trp-dependent auxin biosynthesis led to metabolic differences, auxin biosynthesis pathway intermediates were quantified in the dormant and after-ripened seeds during imbibition (Fig. 4d; Table S6). IAA in dry seeds is mainly stored as conjugates, which are hydrolyzed during early imbibition to yield free IAA (Ljung *et al.*, 2001). The hydrolysis of the IAA conjugates is a rapid process that allows immediate access of IAA to remove the need for long-distance transport. This process is followed by Trp-dependent auxin biosynthesis in developing tissues in the root and shoot apex (Ljung *et al.*, 2001, 2005). Concentrations of free IAA and the conjugates IAA-Asp and IAA-Glu are rather low (50-fold lower) compared with the IAA precursors (indole-3-pyruvic acid (IpyA) and Trp). Free IAA concentrations were only marginally influenced by imbibition time ($P = 0.048$) and no difference was detected between the dormancy stages. The concentrations of Trp and derived IPyA, which are the main Trp-dependent auxin synthesis precursors (Ljung, 2013), were increased in the after-ripened seeds. Interestingly, the auxin influx carrier AUX1 was recently reported to have a positive role in seed germination regulated by histone H3K9K18 deacetylation (Wang *et al.*, 2016). Moreover, the cell cycling-related Cyclin D-type (*CYCD*) genes that are downstream of AUX1 and are important for radicle promotion (Wang *et al.*, 2016) were also among the dormancy and cordycepin-inhibited genes in our analyses (Table S5). This might be a downstream effect of the auxin pathway as auxin is known to influence the cell division in both embryonic and vegetative stages (De Veylder *et al.*, 2007).

In all, our data indicate the importance of auxin biosynthesis in seed germination; however, how the auxin synthesis pathway is activated remains unknown. In-depth studies on the imbibition of seeds at different after-ripening stages could provide insights into this regulation.

Acknowledgements

This work was supported by the Netherlands Organization for Scientific Research (project no. 821.02.010) and Bio4Energy, a Strategic Research Environment appointed by the Swedish government, the Swedish Research Council (VR) and the Swedish Governmental Agency for Innovation Systems (VINNOVA). We thank Service XS, Leiden, the Netherlands, for performing the microarray hybridizations. We thank Harro Bouwmeester and Sjef Smeekens for valuable suggestions on the manuscript.

Author contributions

B.B., J.H. and L.B. designed the experiments. B.B. performed the experiments and conducted the analyses. O.N. and K.L. performed IAA metabolite profiling and analyzed the data. B.B., J.H. and L.B. wrote the manuscript. All authors commented on the manuscript.

References

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M. 2003. Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics* **164**: 711–729.

Bai B, Peviani A, van der Horst S, Gamm M, Snel B, Bentsink L, Hanson J. 2016. Extensive translational regulation during seed germination revealed by polysomal profiling. *New Phytologist* **214**: 233–244.

Bak S, Feyereisen R. 2001. The involvement of two p450 enzymes, CYP83B1 and CYP83A1, in auxin homeostasis and glucosinolate biosynthesis. *Plant Physiology* **127**: 108–118.

Barlier I, Kowalczyk M, Marchant A, Ljung K, Bhalerao R, Bennett M, Sandberg G, Bellini C. 2000. The *SUR2* gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. *Proceedings of the National Academy of Sciences, USA* **97**: 14819–14824.

Basbouss-Serhal I, Pateyron S, Cochet F, Leymarie J, Bailly C. 2017. 5′ to 3′ mRNA decay contributes to the regulation of Arabidopsis seed germination by dormancy. *Plant Physiology* **173**: 1709–1723.

Basbouss-Serhal I, Soubigou-Taconnat L, Bailly C, Leymarie J. 2015. Germination potential of dormant and nondormant Arabidopsis seeds is driven by distinct recruitment of messenger RNAs to polysomes. *Plant Physiology* **168**: 1049–1065.

Bazin J, Langlade N, Vincourt P, Arribat S, Balzergue S, El-Maarouf-Bouteau H, Bailly C. 2011. Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *The Plant Cell* **23**: 2196–2208.

Bentsink L, Hanson J, Hanhart CJ, Blankestijn-de Vries H, Coltrane C, Keizer P, El-Lithy M, Alonso-Blanco C, de Andres MT, Reymond M *et al.* 2010. Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways. *Proceedings of the National Academy of Sciences, USA* **107**: 4264–4269.

Bentsink L, Jowett J, Hanhart CJ, Koornneef M. 2006. Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **103**: 17042–17047.

Bentsink L, Koornneef M. 2008. Seed dormancy and germination. *Arabidopsis Book* **6**: e0119.

Bewley JD. 1997. Seed germination and dormancy. *The Plant Cell* **9**: 1055–1066.

Browning KS, Bailey-Serres J. 2015. Mechanism of cytoplasmic mRNA translation. *Arabidopsis Book* **13**: e0176.

Cadman CS, Toorop PE, Hilhorst HW, Finch-Savage WE. 2006. Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant Journal* **46**: 805–822.

Chibani K, Ali-Rachedi S, Job C, Job D, Jullien M, Grappin P. 2006. Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiology* **142**: 1493–1510.

De Veylder L, Beeckman T, Inze D. 2007. The ins and outs of the plant cell cycle. *Nature Reviews Molecular Cell Biology* **8**: 655–665.

Diboun I, Wernisch L, Orengo CA, Koltzenburg M. 2006. Microarray analysis after RNA amplification can detect pronounced differences in gene expression using limma. *BMC Genomics* **7**: 252.

El-Maarouf-Bouteau H, Meimoun P, Job C, Job D, Bailly C. 2013. Role of protein and mRNA oxidation in seed dormancy and germination. *Frontiers in Plant Sciences* **4**: 77.

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G. 2006. Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. *Plant Physiology* **142**: 839–854.

Finch-Savage WE, Cadman CS, Toorop PE, Lynn JR, Hilhorst HW. 2007. Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. *Plant Journal* **51**: 60–78.

Gamm M, Peviani A, Honsel A, Snel B, Smeekens S, Hanson J. 2014. Increased sucrose levels mediate selective mRNA translation in Arabidopsis. *BMC Plant Biology* **14**: 306.

Gao F, Rampitsch C, Chitnis VR, Humphreys GD, Jordan MC, Ayele BT. 2013. Integrated analysis of seed proteome and mRNA oxidation reveals distinct post-transcriptional features regulating dormancy in wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal* **11**: 921–932.

Gautier L, Cope L, Bolstad BM, Irizarry RA. 2004. affy–analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **20**: 307–315.

Graeber K, Linkies A, Steinbrecher T, Mummenhoff K, Tarkowska D, Tureckova V, Ignatz M, Sperber K, Voegele A, de Jong H *et al.* 2014. *DELAY OF GERMINATION 1* mediates a conserved coat-dormancy mechanism for the temperature- and gibberellin-dependent control of seed germination. *Proceedings of the National Academy of Sciences, USA* **111**: E3571–E3580.

Hemm MR, Ruegger MO, Chapple C. 2003. The Arabidopsis *ref2* mutant is defective in the gene encoding CYP83A1 and shows both phenylpropanoid and glucosinolate phenotypes. *The Plant Cell* **15**: 179–194.

Hofmann N. 2014. Cryptochromes and seed dormancy: the molecular mechanism of blue light inhibition of grain germination. *The Plant Cell* **26**: 846.

Holbein S, Wengi A, Decourty L, Freimoser FM, Jacquier A, Dichtl B. 2009. Cordycepin interferes with 3′ end formation in yeast independently of its potential to terminate RNA chain elongation. *RNA* **15**: 837–849.

Huang X, Schmitt J, Dorn L, Griffith C, Effgen S, Takao S, Koornneef M, Donohue K. 2010. The earliest stages of adaptation in an experimental plant population: strong selection on QTLs for seed dormancy. *Molecular Ecology* **19**: 1335–1351.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Joosen RV, Kodde J, Willems LA, Ligterink W, van der Plas LH, Hilhorst HW. 2010. GERMINATOR: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. *Plant Journal* **62**: 148–159.

Juntawong P, Bailey-Serres J. 2012. Dynamic light regulation of translation status in *Arabidopsis thaliana*. *Frontiers in Plant Sciences* **3**: 66.

Kim JI, Dolan WL, Anderson NA, Chapple C. 2015. Indole glucosinolate biosynthesis limits phenylpropanoid accumulation in *Arabidopsis thaliana*. *The Plant Cell* **27**: 1529–1546.

Klein M, Papenbrock J. 2009. Kinetics and substrate specificities of desulfo-glucosinolate sulfotransferases in *Arabidopsis thaliana*. *Physiologia Plantarum* **135**: 140–149.

Koornneef M, Bentsink L, Hilhorst H. 2002. Seed dormancy and germination. *Current Opinion in Plant Biology* **5**: 33–36.

Layat E, Leymarie J, El-Maarouf-Bouteau H, Caius J, Langlade N, Bailly C. 2014. Translatome profiling in dormant and nondormant sunflower (*Helianthus annuus*) seeds highlights post-transcriptional regulation of germination. *New Phytologist* **204**: 864–872.

Leon RG, Owen MDK. 2003. Regulation of weed seed dormancy through light and temperature interactions. *Weed Science* **51**: 752–758.

Lin SY, Chen PW, Chuang MH, Juntawong P, Bailey-Serres J, Jauh GY. 2014. Profiling of translatomes of in vivo-grown pollen tubes reveals genes with roles in micropylar guidance during pollination in Arabidopsis. *The Plant Cell* **26**: 602–618.

Liu Y, Ye N, Liu R, Chen M, Zhang J. 2010. H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in Arabidopsis seed dormancy and germination. *Journal of Experimental Botany* **61**: 2979–2990.

Ljung K. 2013. Auxin metabolism and homeostasis during plant development. *Development* **140**: 943–950.

Ljung K, Hull AK, Celenza J, Yamada M, Estelle M, Normanly J, Sandberg G. 2005. Sites and regulation of auxin biosynthesis in Arabidopsis roots. *The Plant Cell* **17**: 1090–1104.

Ljung K, Ostin A, Lioussanne L, Sandberg G. 2001. Developmental regulation of indole-3-acetic acid turnover in Scots pine seedlings. *Plant Physiology* **125**: 464–475.

Mach J. 2015. Metabolic crosstalk: interactions between the phenylpropanoid and glucosinolate pathways in Arabidopsis. *The Plant Cell* **27**: 1367.

Meimoun P, Mordret E, Langlade NB, Balzergue S, Arribat S, Bailly C, El-Maarouf-Bouteau H. 2014. Is gene transcription involved in seed dry after-ripening? *PLoS ONE* **9**: e86442.

Missra A, Ernest B, Lohoff T, Jia Q, Satterlee J, Ke K, von Arnim AG. 2015. The circadian clock modulates global daily cycles of mRNA ribosome loading. *The Plant Cell* **27**: 2582–2599.

Mustroph A, Juntawong P, Bailey-Serres J. 2009a. Isolation of plant polysomal mRNA by differential centrifugation and ribosome immunopurification methods. *Methods in Molecular Biology* **553**: 109–126.

Mustroph A, Zanetti ME, Jang CJ, Holtan HE, Repetti PP, Galbraith DW, Girke T, Bailey-Serres J. 2009b. Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **106**: 18843–18848.

Novak O, Henykova E, Sairanen I, Kowalczyk M, Pospisil T, Ljung K. 2012. Tissue-specific profiling of the *Arabidopsis thaliana* auxin metabolome. *Plant Journal* **72**: 523–536.

Novak O, Pěnčík A, Blahoušek O, Ljung K. 2016. Quantitative auxin metabolite profiling using stable isotope dilution UHPLC-MS/MS. *Current Protocols in Plant Biology* **1**: 419–430.

Ouyang J, Shao X, Li J. 2000. Indole-3-glycerol phosphate, a branchpoint of indole-3-acetic acid biosynthesis from the tryptophan biosynthetic pathway in *Arabidopsis thaliana*. *Plant Journal* **24**: 327–333.

Pfalz M, Mikkelsen MD, Bednarek P, Olsen CE, Halkier BA, Kroymann J. 2011. Metabolic engineering in *Nicotiana benthamiana* reveals key enzyme functions in Arabidopsis indole glucosinolate modification. *The Plant Cell* **23**: 716–729.

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D. 2012. Seed germination and vigor. *Annual Reviews in Plant Biology* **63**: 507–533.

Rajjou L, Gallardo K, Debeaujon I, Vandekerckhove J, Job C, Job D. 2004. The effect of alpha-amanitin on the Arabidopsis seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination. *Plant Physiology* **134**: 1598–1613.

Smyth GK. 2005. *Limma: linear models for microarray data*. New York, NY, USA: Springer.

Subramanian V. 1978. Polyribosome formation during early germination of bean embryonic axes. *Indian Journal of Biochemistry Biophysics* **15**: 235–237.

Sugawara S, Hishiyama S, Jikumaru Y, Hanada A, Nishimura T, Koshiba T, Zhao Y, Kamiya Y, Kasahara H. 2009. Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **106**: 5430–5435.

Sun JQ, Xu YX, Ye SQ, Jiang HL, Chen Q, Liu F, Zhou WK, Chen R, Li XG, Tietz O *et al.* 2009. Arabidopsis *ASA1* is important for jasmonate-mediated regulation of auxin biosynthesis and transport during lateral root formation. *The Plant Cell* **21**: 1495–1511.

Vragovic K, Sela A, Friedlander-Shani L, Fridman Y, Hacham Y, Holland N, Bartom E, Mockler TC, Savaldi-Goldstein S. 2015. Translatome analyses capture of opposing tissue-specific brassinosteroid signals orchestrating root meristem differentiation. *Proceedings of the National Academy of Sciences, USA* **112**: 923–928.

Wang Z, Chen FY, Li XY, Cao H, Ding M, Zhang C, Zuo JH, Xu CN, Xu JM, Deng X *et al.* 2016. Arabidopsis seed germination speed is controlled by SNL histone deacetylase-binding factor-mediated regulation of *AUX1*. *Nature Communications* **7**: 13412.

van Zanten M, Koini MA, Geyer R, Liu YX, Brambilla V, Bartels D, Koornneef M, Fransz P, Soppe WJJ. 2011. Seed maturation in *Arabidopsis thaliana* is characterized by nuclear size reduction and increased chromatin condensation. *Proceedings of the National Academy of Sciences, USA* **108**: 20219–20224.

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL. 2002. Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. *Genes & Development* **16**: 3100–3112.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Gene 1.1 ST Genechip quality assessment and reproducibility.

Fig. S2 Absorbance profiles of sucrose density gradient fractionated ribosomes at 0, 6 and 24 h after imbibition (HAI).

Fig. S3 Bar graph representing the number of total and polysomal transcripts influenced and inhibited by cordycepin and seed dormancy.

Fig. S4 Venn diagram comparing total and polysomal transcripts influenced by dormancy.

Fig. S5 Confirmation of the cordycepin effect on changes in transcript abundance during seed imbibition.

Table S1 The normalized and filtered dataset used for statistical analysis

Table S2 Gene ontology for the total and polysomal RNA changes affected by seed dormancy

Table S3 Gene ontology for the transcriptional changes during dormant seed imbibition in water and nondormant seed imbibition in cordycepin

Table S4 Gene ontology for the genes affected by both dormancy and cordycepin

Table S5 Genes affected by both seed dormancy and cordycepin

Table S6 Concentrations of IAA and IAA precursors/metabolites in pg mg^{-1} DW

Table S7 Genes and primers used for qRT-PCR

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.